Annals of the Royal College of Surgeons of England (1984) *vol.* 66

Mechanisms of tissue destruction following cryosurgery*

D K WHITTAKER PhD FDS RCS
Reader and Honorary Consultant Dental Surgeon
Welsh National School of Medicine

Key words: CRYOSURGERY; ORAL MUCOSA; ICE-CRYSTALS; ELECTRON MICROSCOPY; NERVES; MICROCIRCULATION

Summary

Destruction of diseased tissue in situ by means of freezing is well established in many branches of surgery. The tissues are apparently unaltered at thaw but progressive necrosis ensues. There is controversy as to whether tissue death is principally due to the direct effects of freezing or to subsequent ischaemia.

Studies at the ultrastructural level show that ice-crystals are formed within the cells during cryosurgery, that resultant cell damage is osmotic rather than mechanical and that microcirculatory changes are secondary in terms of the chronological development of tissue necrosis.

Introduction

Although freezing techniques have only recently become widespread as a surgical tool, medical literature has recorded the use of sub-zero temperatures since the time of the earliest records. Probably the earliest known example of medical literature is the Edwin Smith Surgical Papyrus written in about 3500 BC and translated into English by Breastead *(1)*. Few details are given in this manuscript, but cold compresses were used to treat fractures of the skull and other battle wounds. A similar approach was recorded by Homer in the Iliad and it was recommended by Hippocrates that cold be used to check severe haemorrhage and reduce post injury swelling.

It was recorded by the surgeon to Napoleon that amputations could be carried out painlessly if the affected limb had been sufficiently cooled in the snow prior to the operation. In the mid 19th century Richardson *(2)* carried this observation further by introducing the ether spray as a local anaesthetic agent, modifications of which, in the form of ethyl chloride are still in common use.

It was not until somewhat lower temperatures could easily be achieved that it was realised that freezing could destroy tissue and hence be of direct use in surgery. An Englishman named Arnott *(3)* was the first physician to make use of this property and he devised instruments for producing and applying temperatures down to $-12\,^{\circ}C$ for the destruction of cancerous growths of the skin. Before the turn of the century, as low temperature physics was advancing, other means of freezing tissues were examined.

The development of cryosurgery largely progressed on a clinical basis and interest in the mechanism of tissue destruction lagged behind the purely technical considerations.

The majority of the early reports were concerned with superficial applications of freezing agents to the skin. By about 1961 the engineering problems of producing low temperature probes were beginning to be solved largely by impetus of the neurosurgeons and these advances have undoubtedly led to the popularity of cryosurgery not only in the dermatological, neurological and ophthalmic fields but in many other branches of surgery.

Present instruments are of three basic types. The first are those using evaporation or change of phase to produce low temperature. The second type use the thermoelectric effect and the third type employ adiabatic expansion of a compressed gas.

This latter approach appears to offer the greatest potential for further development.

Application in the oral cavity

The first applications of cryosurgery in or around the mouth appear to have been some trials carried out on the treatment of cancer of the lip and oral cavity *(4)*. These workers quickly came to the conclusion that extensive bone-sacrificing operations could be avoided and the technique was especially suitable for poor risk patients. Healing of the cryosurgical wounds in the mouth left little scarring. In these early stages it was not possible to estimate the long term effects of the treatment. In 1969 the use of liquid nitrogen treatment for oral and oro-pharyngeal cancers in a group of 50 patients was reported. Previous work on the freezing of large blood vessels in dogs allowed the suggestion that freezing of major vessels close to tumours was not to be feared.

In connection with the treatment of oral carcinoma by means of cryotherapy, Poswillo *(5)*, discussed the advantages of cryotherapy over other conventional methods and stated that even if cartilage, bone and large blood vessels are involved they will retain their skeletal functions until the dead cells are replaced. He concluded by suggesting that the technique will become an effective addition in the armamentarium of the oral surgeon and may have additional advantages of controlling pain and perhaps dealing with lesions in sites that would be difficult to treat by other means.

A more experimental approach in which surgically produced hyperplastic lesions in the buccal sulci of monkeys were destroyed either by electrosurgery or by cryosurgery *(6)* indicated that healing, both in the case of cryosurgery and electrosurgery was slower than following excision but there was eventually less scar formation. Recent reports *(7)* have confirmed the effectiveness of cryosurgery for pre-malignant oral lesions.

In connection with the treatment of intra-bony oral lesions experiments were carried out on the freezing of canine mandibles by reflecting soft tissue and circulating liquid nitrogen through tubes wrapped around them *(8)*. Current methods of treating bone lesions have been described in detail recently *(9)*.

Odrich and Kelman *(10)* were the first workers to use cryotherapy for the treatment of periodontal disorders. From experience gained by ophthalmologists these writers argued that the application of low temperatures in the region of periodontal pockets might produce adhesion of the detached tissue by means of an inflammatory exudate.

* Based on a Hunterian Lecture delivered in Cardiff, February 1983

The Editor would welcome any comments on this paper by readers

The only report of the treatment of infectious diseases of the mouth by means of cryotherapy appears to be that by Krashen *(11)* who studied the effect of freezing on herpes of the mouth. Following earlier satisfactory reports of treatment of herpes simplex keratitis and experimental work in which the concentration of viruses was markedly reduced by rapid freezing and thaw, this author applied carbon dioxide snow to the lesion for 6 seconds repeated on 3 occasions. Morbidity was reduced to 2 to 3 days and no recurrences were reported.

A review of cryosurgery of benign oral neoplasms *(12)* concluded that the technique is particularly suitable for the treatment of vascular or angiomatous lesions, lesions involving bone and for the treatment of poor risk patients. The view was expressed that cryosurgery is painless because of the immediate destruction of nerve endings in the area and this clinical observation has recently been utilised in the treatment of intractable facial pain by means of cryosurgical intervention *(13)*. Pain was successfully relieved in 5 of the 6 patients.

A number of reviews of the uses of cryosurgery in the mouth have been published *(14–16)* and the last decade has seen a proliferation of clinical reports on the use of cold therapeutics in dentistry. This acceptance of a new technique has depended largely on the scientific background of freezing studies carried out under different experimental conditions. The present study is an attempt to investigate some of these parameters under conditions more closely allied to the clinical situation.

Ice-crystals in living tissues

The demonstration of ice-crystals in cornea frozen under conditions for cryosurgery *(17)* appears to be the only direct evidence for the assertion that ice-crystals formed either intra or extra cellularly are responsible for cell death after low temperature destruction of tissue in in-vivo circumstances as distinct from laboratory conditions.

It has been suggested *(18)* that such crystals will be mainly extracellular following the freezing rates used in clinical cryosurgery but there is no experimental evidence to confirm this view. The importance of further investigation in this field has been recognised but there have been few rigorous studies carried out on tissues and organs.

Cryosurgery of the ventral mucosa of the tongue of golden hamsters was carried out for 1 minute at a tip temperature of −70 °C. Portions of mucosa were excised whilst still frozen and attached to the probe tip and the frozen specimens were split off from the probe and processed by freeze drying or freeze substitution for examination in the AEI EM6B transmission electron microscope.

In the cryoprobed tissues variation in size of ice crystal spaces (ICS) and their distribution depended upon the proximity to the freezing probe, the type of tissue and whether the tissue possessed an intact blood supply at the time of freezing or had been excised from the animal. Within the lesion centre and immediately deep to the probe the epithelium contained ICS throughout the tissue. These were multi-locular in outline and were apparently all intracellular. There was no evidence of extracellular crystals distorting the cells although cell boundaries were difficult to distinguish. Between the ICS were electron dense septa which on higher magnification were seen to contain distorted cytoplasmic constituents.

Within the cell nuclei the ICS were larger and each nucleus contained only 2 or 3 lobed ICS. The nuclear material was compressed between the lobes of the ICS.

In the muscle fibres within the lesion area, the distribution of ICS was less regular. In approximately half the fibres the spaces were small and oval in shape. There was only slight evidence of muscle tissue between the ICS. In the remaining fibres ICS were of a similar shape but of much larger dimensions (Fig. 1). These spaces were still within the muscle fibres but detail of structure could be distinguished between

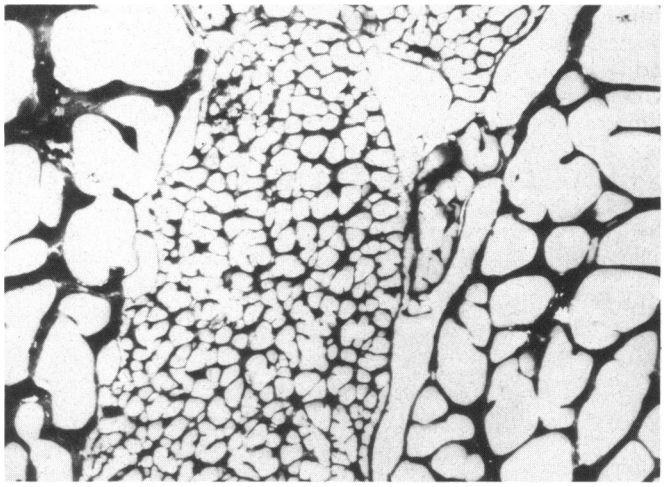

FIG. 1 Muscle fibres frozen by means of a cryoprobe at −70 °C in the living animal. Intracellular ice-crystals (ICS) are large in some fibres and small in adjacent fibres. × 10 000.

the ice artefacts. At higher magnification the myofilament pattern was distinguishable, although distorted, and mitochondria were structurally intact.

Ice-crystal pattern in epithelium from tissues at the periphery of the ice-ball differed from that in the area of the probe. ICS were almost exclusively extracellular and large. A few small spaces were present within the epithelial cytoplasm and nuclei but these were rare. The extracellular ice could be seen to be producing severe compression and distortion of the cells but at higher magnification, cell cytoplasm was relatively intact and cell organelles were visible (Fig. 2).

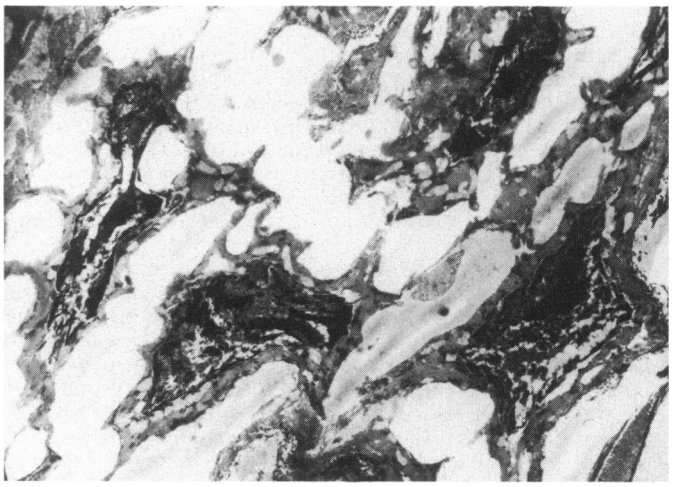

FIG. 2 ICS in epithelium at periphery of frozen area. Crystals are mainly extracellular resulting in compression of cells. × 10 000.

A similar pattern existed in the muscle fibres in this zone where ice-crystal spaces were all extracellular. The muscle fibres were distorted in general outline but the normal constituents of the fibres were clearly visible.

In tissues subjected to cryosurgery after excision from the living animal the ice-crystal sizes were intermediate between those of the control animals and those of the animals cryoprobed in vivo. The dimensions of ICS in any particular cell were regular.

In the underlying muscle fibres ICS were also smaller than those in the tissues cryoprobed in the intact animal.

Unlike the situation in the intact animal, the spaces were regular in size and distribution in most of the muscle fibres studied.

No previous studies have been carried out on the ultrastructural appearance of ice-crystal artefacts in tissues

subjected to freezing by means of a surface applied cryosurgical probe. The present experiments were designed to indicate whether the ice-crystals were intra or extracellular, what their relationship was to cell structures and what their dimensions were. Evidence of penetration of cell organelles or cell membranes by ice-crystals was also sought.

There was no evidence that penetration of cell membranes by ice crystals had occurred. The appearances were of grossly distorted cytoplasm produced by compression. The theory of cell damage during freezing *(19)* which postulates rupture of membranes by rapid passage of water is therefore not substantiated. Moreover this theory is based on the assumption that the intracellular water remains liquid below O °C and the present experiments clearly demonstrate that this does not occur.

The variation in size of ice-crystals in muscle fibres in the central part of the lesion is confirmed. The small ICS seen in approximately 50% of fibres resulted in loss of structural detail in the muscles and no intact intracellular organelles were visible. The large ICS however had clearly compressed the normal architecture but myofilaments and intact mitochondria were seen at high magnification. The degree of damage to the fibres appears to depend therefore not only on whether the crystals are extra or intracellular but also on their size. It is suggested that this provides an explanation of the diversity of opinion on whether intracellular ice is always lethal *(17,20)*.

The variation in susceptibility of muscle fibres to ice-crystal formation is believed to be due to regional difference in the microcirculation surrounding the fibres. The evidence for this assumption is based on the finding that ice-crystals in muscle fibres cryoprobed after excision from the animal were not only smaller than in the in-vivo situation, but were also regular in size. It is suggested that apart from the role played by the microvasculature in the development of postcryosurgery ischaemia it may also contribute substantially to the local thermal properties of the tissues.

Effect of repeat freezes

Many clinicians have stressed the importance of careful control of the parameters of freezing if maximum tissue destruction is to be obtained. One such parameter is the number of applications of a freezing probe to the tissue to be treated but the reason for the efficacy of a repeat freeze regimen is not clear. Some workers *(21)* postulate that the initial freeze produces an increase in thermal conductivity which results in a more effective second freeze. If this is the case then repeat freezes should result in smaller intracellular ice-crystals due to the more rapid freeze consequent upon better thermal conductivity.

Specimens from animals subjected to single freezes and those subjected to double freezes with thaw periods ranging between 5 and 30 minutes were freeze substituted for transmission electron microscopy.

Ice crystal spaces in the epithelium and muscle fibres of the tissues with interfreeze periods of 5 and 10 minutes were regular in size throughout the central zone of the ice-ball.

In the tissues thawed for 10 minutes between freezes the ICS were also regular in size in the muscle fibres but larger than in the previous group.

In the material allowed to thaw for 30 minutes between freezes the ICS were usually of a regular size in most of the muscle fibres but some fibres contained very large ice-crystals which appeared to have disrupted the structure of the fibre.

An increase in mean diameters of the ICS with increased interfreeze periods was demonstrated. This increase in size is unexpected in view of the increased thermal conductivity in tissues caused by the first freeze. This should result in a more rapid freeze and therefore smaller ICS. The fact that it does not suggests that some other factor has a greater influence on freezing rate. It seems likely that an increased heat sink due to dilated vessels may be responsible. This heat sink would result in a slower freezing rate and therefore larger ice-crystals would be formed.

In order to explain the clinical findings it would have to be postulated that larger intracellular ice-crystals are more lethal than smaller ones, but there is still disagreement about this matter. From a clinical viewpoint the results imply that as long a period as possible should be left between repeat freezes providing that the time does not exceed that at which stasis of the microcirculation intervenes.

Early cellular changes in epithelium

The mechanisms of cell death following freezing have received considerable attention. Studies of isolated cells or tissues have defined ice-crystal dimensions and locations and have led to the commonly held view that intracellular ice is usually lethal, whereas extracellular ice is not *(22)*. Ischaemia due to microvascular stasis has been shown to occur following frost-bite injuries but there is no general agreement as to the relative importance of direct damage to tissues by ice-crystals or subsequent necrosis caused by vascular events. Few studies have been carried out under clinical conditions of freezing and chronological investigations commencing immediately on thaw do not appear to have been reported.

It has been shown by previous workers that ischaemia does not normally develop for some time after surface freezing *(23)* and the present investigation is of early epithelial changes following freezing and thawing in the interval before ischaemic changes would be expected to be of importance.

Ventral epithelium of the tongue of golden hamsters was frozen at −70 °C for 1 minute. Animals were sacrificed immediately and at various time intervals postoperatively. Specimens were prepared for transmission electron microscopy.

Progressive changes in the cytoplasmic constituents, cell wall structure, lamina densa and nucleus were apparent following thaw.

Immediately on thaw mitochondria were intact although somewhat swollen. There was dilatation of some sections of the endoplasmic reticulum but the distribution of ribosomes, the structure of tonofilaments and desmosomes and the width of the intercellular spaces appeared normal. In some of the nuclei examined at this stage electron lucent areas were present due to the presence of ice-crystals.

Two minutes after thaw, small intracytoplasmic vesicles bounded by a double membrane were seen. Large vacuoles were also present and serial sections disclosed that these were in continuity with the extracellular spaces and were causing nuclear compression. The widened intercellular spaces of the basal cells contained membrane bounded granular structures which were continuous with the cytoplasm of the cells. Most cell membranes were intact at this stage but pseudopodia-like extensions of the cell appeared to invaginate the cell wall and cytoplasm of adjacent cells. Desmosomal attachments were intact. Tonofilaments were of normal structure but slightly less densely arranged than in the controls.

The lamina densa was intact but there was an increase in width of the lamina lucida compared to the control tissue. The enlarged intercellular spaces were spanned by processes of some basal cells. There was marked distortion of nuclei at this stage because of pressure from developing vacuoles and increased clumping of chromatin material at the nuclear periphery had occurred.

In the 30 minutes postoperative material mitochondrial damage was observed with loss of density of the matrix and distortion or loss of cristae. In those mitochondria where cristae loss was complete, their membranes enclosed a flocculent material. In some cases disruption of the outer membranes of these organelles was apparent or they were widely spaced.

Cytoplasmic density was reduced and in some cells ribosomes were widely spaced and tonofilaments were

sparsely arranged rather than in dense bundles. Loss of definition of cell walls was a constant feature and involved the plasma membranes of adjacent cells so that merging of cytoplasmic contents across intercellular spaces appeared to be occurring.

Basal cell attachment to the lamina densa was affected although in many cells the area of damage was localised and consisted of a loss of cell membranes and disappearance of hemidesmosomes. There was a diminution of electron density of the central area of the nucleus but the peripheral chromatin pattern remained similar to that seen at 10 minutes postoperatively.

In material examined 2 hours postoperatively two distinct methods of formation of bullae were present. Vacuoles developing intracytoplasmically were seen in some cells close to the lamina densa.

The other method of bullous formation appeared to be due to an extension of the previously described withdrawal of hemidesmosomes from the lamina densa which, however, remained intact (Fig. 3).

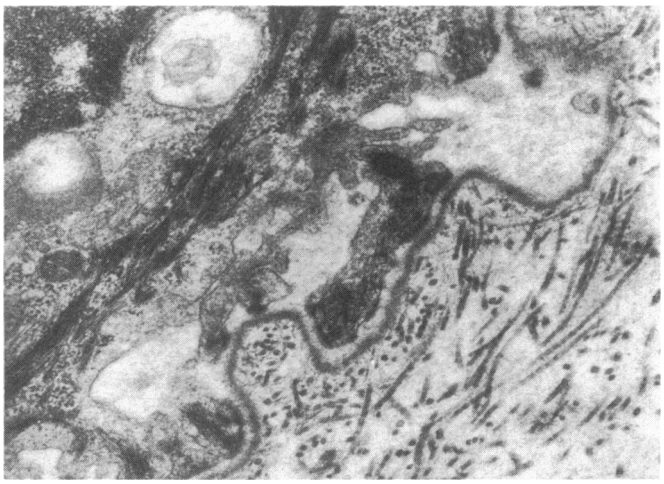

FIG. 3 Basal cells of epithelium 2 hours after thaw showing retraction of hemidesmosomes from lamina densa. Lamina densa remains intact. × 40 000.

The nuclei were smaller in size and more rounded in outline and there was evidence of disruption of chromatin material at the periphery of the structure as well as centrally.

By the 5-hour stage cell necrosis was advanced so that cellular detail was increasingly difficult to recognise. The dimensions of bullae in the basal zone had increased and detachment of the basal cells from the lamina densa was more extensive. Nuclei were still present amongst the remnants of the basal cells but they were pyknotic.

It is evident from this study that epithelium immediately on thaw bears close similarity to normal tissue. The only changes were slight irregularity of the nuclear membrane and chromatin pattern and some dilatation of mitochondria, cell vesicles and endoplasmic reticulum.

These changes provide no evidence for rupture of cell and organelle membrane systems so that direct puncture of membranes by ice-crystals, rupture via contraction of cells or by sudden loss of intracellular water seem to be unlikely following the rates of freezing and thawing used. Clearly demonstrable post freezing and thawing changes in the epithelial cells did not become evident until 2 minutes after thaw. Even after this time had elapsed cell membranes remained intact although there was a steadily increasing loss of definition of membranes as time progressed. The appearance of pseudopodia of epithelial cells invaginating the cytoplasm of adjacent cells is an unusual one which does not appear to have been reported previously following epidermal damage. It is known from studies of healing epidermal wounds that basal epithelial cells commence to migrate across the wound defect by means of extending pseudopodia *(24)* and it may be that damage and disruption of some epithelial cells following freezing and thawing provides the stimulus for less damaged cells to proliferate in this way.

The first changes seen in the region of the dermo–epidermal junction of this study consisted of a widening of the lamina lucida occasioned by movement of the plaques of the hemidesmosomes away from the lamina densa. Studies of the ultrastructure of cell attachment and lamina densa topography suggest that separation of cells in these two sites could theoretically be due to retraction of tonofilaments resulting in loss of hemidesmosomes or desmosomes, damage to plasma membranes themselves or some changes in the mucopolysaccharide coating of the cells or intercellular attachment areas.

No evidence of cell membrane disruption, loss of tonofilaments or changes in lamina densa were seen in these early stages. It seems likely therefore that bullous formation results either from osmotic forces overcoming the attachment nature of the lamina lucida or from changes in the adhesive nature of the lamina lucida during the freezing and thawing process.

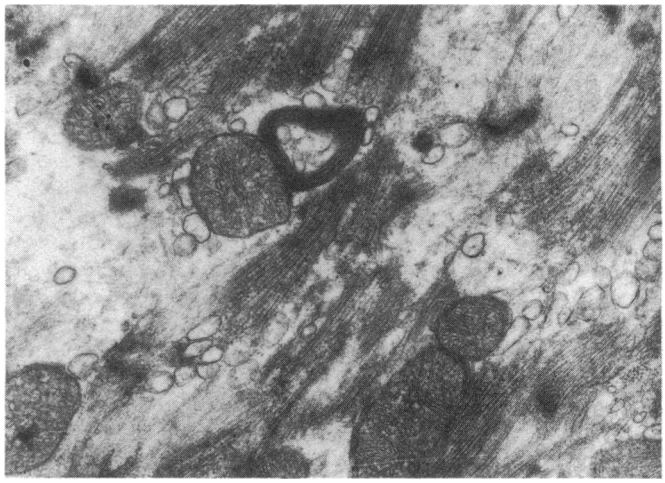

FIG. 4 Severely damaged muscle fibre 2 hours after thaw. Note collections of myofilaments with irregular arrangement and no evidence of banding. × 30 000.

Early cellular changes in muscle

The majority of cell organelles were intact immediately on thaw and the most marked changes involved the mitochondria and the Z lines. All the muscle fibres in the central area of the lesion appeared to be effected to the same degree.

There was irregularity of the Z and I bands which pursued a wavy course and in some sites were absent.

Mitochondria were enlarged with decreased matrix density and disruption of cristae although the outer membranes remained intact. Ten minutes after thaw a variable response of the individual muscle fibres to freezing was seen. Some fibres were almost indistinguishable from normal and the structure of the myofilaments and their characteristic banding was demonstrable although not so clearly demarcated into the A and I zones..

In the more severely damaged fibres, mitochondria were effected to a greater degree than in material examined at thaw, or in the lesser damaged fibres described above. Sarcoplasmic reticulum between the myofilaments was more obvious and this was largely due to distension of vesicles. The arrangement of actin and myosin filaments appeared to be unchanged. Glycogen was less abundant than in the immediately on thaw specimens.

One hour after freezing the presence of both mildly and severely damaged fibres was noted.

The normal banded appearance of the myofibrils had disappeared although some retained a somewhat denser zone in the central area reminiscent of Z band. Mitochondria

were variable in structure and both the small dense variety and larger swollen and disrupted organelles were present. No evidence of glycogen particles was seen in any of the sections examined.

At 2 hours dissolution of most muscle bundles had progressed to such an extent that only remnants of the normal structure were retained (Fig 4). A striking feature of the resistant fibres was the lack of disruption of mitochondria which although swollen retained a cristae pattern which was dilated but intact.

By 5 hours postoperatively the less severely damaged fibres possessed mitochondria at their periphery which were intact. The most severely damaged fibres were filled with a loosely arranged flocculent material bounded by an intact lamina densa. The loss of glycogen from skeletal muscles following ischaemia is well documented in both biochemical and histochemical studies but does not become apparent for 8–11 hours. In the present material some loss was apparent immediately on thaw and by 1 hour glycogen had disappeared completely from the sections. This time scale is more comparable to that seen after crushing injuries of muscle.

The later changes involved increasingly severe mitochondrial damage. Mitochondrial changes are a well documented feature of most pathological changes in muscle and they are important because of the general agreement that the entire mechanism of oxidative phosphorylation is located in the mitochondrial membranes. It seems reasonable to assume that the structural degradation of mitochondria seen in the muscle subjected to cryosurgery would result in impairment if not in cessation of function.

Previous work has demonstrated that the Z line is susceptible to ischaemia and this condition is an interesting one to compare with the present material since ischaemic infarction has been postulated as a cause of cell death following freezing in situ.

The variation in resistance of myofibrils to freezing damage confirms an earlier report at the light microscope level *(25)*. It is unlikely to be due to temperature differences in adjacent fibrils and structural differences or the state of hydration at the time of freezing may be responsible. A further possibility is that variations in blood supply to adjacent fibres may play some role in this phenomenon.

Damage and recovery in nervous tissue

It has generally been assumed that cryosurgery is a painless procedure because of the immediate blockage of nerve transmission in the area. Low temperatures have been shown to be capable of blocking neural transmission but the reversibility of this procedure seems to be under debate.

In comparison to control specimens myelinated nerves in the experimental tissues consistently showed changes which could be attributed to the freeze–thaw injury. Contraction of the axon from the myelin sheath had occurred as indicated by a spacing between the inner leaflet of the sheath and the axolemma.

This axolemma appeared to be intact although in some areas there was poor resolution of the membranes. Mitochondria of the axon were swollen and there was disruption or loss of cristae. There was early evidence of myelin disruption in the sheath as indicated by an increased width between adjacent leaflets resulting in a corrugated appearance.

As time progressed, damage to myelinated nerves was considerable. The cytoplasm of the Schwann cells was less distinct than in earlier specimens and mitochondria were greatly enlarged. The cytoplasm of the Schwann cells was ballooned out into large vacuoles which in some cases had compressed the myelin. There was considerable loss of structure in the cytoplasm of the axons with loss of microtubules and microfilaments.

By 5 hours post-thaw disruption of axons in myelinated nerves was complete and only a finely dispersed flocculent precipitate remained with no evidence of the axolemma. The degree of corrugation and distortion of the myelin sheath was slightly more severe than in the earlier material. Disruption of the Schwann cell cytoplasm had occurred.

It is not possible to say whether this damage is sufficient to account for lack of transmission of nerve impulses and hence a pain-free postoperative situation but taken in conjunction with clinical evidence this appears to be the case.

Changes in the microcirculation

These were examined by injection of carbon particles into the vascular system at various stages after thawing in order to detect permeability. In addition dynamic studies using implanted plastic windows in the hamster cheek pouch were used. In the carbon studies immediately on thaw the vessels appeared normal.

Thirty minutes after thaw there was swelling of the endothelial cells resulting in protrusions into the lumen of the vessel.

In the 1-hour post-thaw material capillaries in both the subepithelial zone and between the muscle bundles were similar in appearance. Their cytoplasm contained large and numerous vacuoles. Defects were observed at the endothelial cell junctions and there was evidence of carbon particles in the lumen. In the more severely damaged capillaries carbon was associated with the endothelial cell membranes and there was evidence of migration of the carbon particles through the vessel walls but not into surrounding tissues.

Muscle capillaries examined from the 2-hour postoperative specimens had a swollen appearance with enlargement and rupture of mitochondria and a less electron-dense appearance in the cytoplasm. Prominent gaps were present between some endothelial cells and carbon particles, when present, were associated with these gaps. The venules examined immediately on thaw and at 10 minutes postoperatively appeared normal.

Venules in animals sacrificed 45 minutes after cryosurgery showed more marked changes. The cytoplasm of the endothelial cells was less dense than in the controls and contained enlarged vesicles. The basement membrane was intact but there were deficiencies in the vessel walls occasioned by the presence of small gaps between the endothelial cells. In most of the sections examined erythrocytes were present in the lumen and platelets were associated with the endothelial gaps. There was no evidence of carbon particle deposit in the majority of vessels examined.

At the $1\frac{1}{2}$-hour stage there were both intraluminal and intramural deposits of carbon present. The particles were usually arranged in clumps and they were either 'trapped' between the other contents of the vessel or were associated with defects in the vessel wall (Fig. 5).

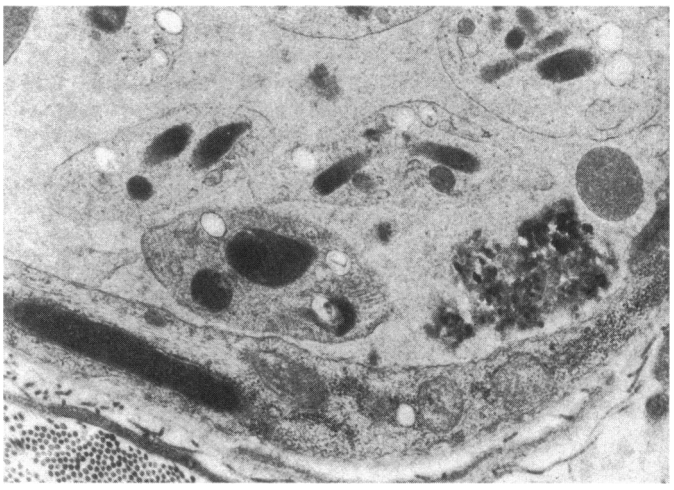

FIG. 5 Venule $1\frac{1}{2}$ hours after thaw. Abundance of platelets within lumen. Carbon associated with vessel wall. × 30 000.

The number of venules containing substantial deposits of carbon was fewer than in the previous group. Gaps between the endothelial cells were larger.

In the final group of specimens injected with carbon 4 hours after freezing a wide range of response of the venules to the freezing episode was visible and did not appear to be related to the anatomical position of the vessel.

A characteristic feature of some venules studied at this time interval was the increasing damage to the endothelial cell walls. Loss of cytoplasmic content and 'ballooning' of endothelial cells was a common finding.

These results indicate that both capillaries and venules in the subepithelial and muscle plexuses are involved in the response to low temperatures in vivo. The development of damage in the capillaries and especially those in the subepithelial zone lagged behind that of the venules so far as endothelial changes were concerned.

The present finding of delayed cytoplasmic damage and the delayed development of intercellular spacing in both capillaries and venules of subepithelial and deeper tissues appears to be unique.

The presence of platelets in vessels damaged by freezing is important in view of their role in the initiation of vessel stasis.

The carbon injection studies have provided some information on the type of permeability change occurring. Carbon could associate itself with damaged vessels either intraluminally or associated with the vessel wall. The presence of intraluminal carbon deposits must be interpreted with some caution, but it is generally agreed that carbon associated with vessel wall defects is a measure of their increased permeability.

The absence of carbon either intraluminally or intramurally from the 5-hour specimens and the presence of erythrocytes and platelets occluding the lumen at this stage is in accord with the development of static thrombosis in these vessels which would prevent the ingress of circulating carbon particles to the site of damage. The lack of carbon labelling is due therefore not to the recovery of the vessels but to their complete blockage in the hours following freezing.

The dynamic studies using hamster cheek pouch chambers confirmed this time scale of events which may be summarised as recovery of flow immediately on thaw, dilatation and increased flow up to about 1 hour postoperatively and then a slowly increasing damage to vessel walls resulting in platelet thrombi and cessation of flow not before 5 hours after operation.

Conclusions

The experiments carried out on intact tissues under conditions similar to those used in clinical cryosurgery have clarified some of the mechanisms involved in tissue destruction.

It has been shown, using freeze substitution and electron microscopy, that ice crystals form intracellularly during cryosurgery although evidence from freezing isolated cells would suggest that the freezing rates used clinically would be more likely to result in extracellular ice. It appears that cells in close contact in living tissues behave differently to those suspended in liquids.

Repeat freezes result in rather large intracellular ice crystals and it is this increase in size which appears to be more lethal following this technique.

The changes occurring in living tissues immediately after thaw have been described both in epithelium and muscle and they are indicative of osmotic damage rather than physical disruption.

Dynamic and permeability studies on the microcirculation suggest that ischaemia is not a problem in the first few hours after thaw and it is concluded that the initial cause of cell damage after cryosurgery is due to the direct effects of the ice crystals within the cells.

References

1 Breastead JH. University of Chicago Oriental Institute Publications. The Edwin Smith Surgical Papyrus 1930;111:217.
2 Richardson BW. On a new and ready mode of producing local anaesthesia. Med Times and Gaz 1866;115:1–7.
3 Arnott J. On the treatment of cancer by the regulated application of an anaesthetic temperature. London: J Churchill 1851;32–54.
4 Gage AA, Koepf S, Wehrle D, Emmings F. Cryotherapy for cancer of lip and oral cavity. Cancer 1965;18:1646–51.
5 Poswillo DE. Cryosurgery and electro surgery compared in the treatment of experimentally induced oral carcinoma. Br Dent J 1971a;131:347–52.
6 Poswillo DE. A comparative study of the effects of electro surgery and cryosurgery in the management of benign oral lesions. Br J Oral Surg 1971b;9:1–7.
7 Al-Drouby HAL. Oral leukoplakia and cryotherapy. Br Dent J 1983;155:124–5.
8 Emmings FG, Neiders ME, Greene GW, Koepf SW, Gage AA. Freezing the mandible without excision. J Oral Surg 1966;24:145–55.
9 Bradley PF. Modern trends in Cryosurgery of bone in the maxillo–facial region. Int J Oral Surg 1978;7:405–15.
10 Odrich R, Kelman CD. Cryotherapy: a new and experimental approach to the treatment of periodontal disease. Periodontics 1967;5:313–17.
11 Krashen AS. Cryotherapy of herpes of the mouth. J Am Dent Assoc 1970;81:1163–5.
12 Feder N, Statigos GT. Current status of cryosurgery for benign oral neoplasms. J Can Dent Assoc 1971;37:146–50.
13 Barnard D, Lloyd J, Evans J. Cryoanalgesia in the management of chronic facial pain. J Maxillofac Surg. 1981;9:101–2.
14 Leopard PJ, Poswillo DE. Practical cryosurgery for oral lesions. Br Dent J 1974;136:185–96.
15 Poswillo DE. Cryosurgery in Oral Surgery. Br J Hosp Med 1975;14:47.
16 Jolly M. Cryosurgery. Aust Dent J 1976;21:35–7.
17 Hanna C, Sherman JK. Survival of rabbit corneal cells after the formation and dissolution of intracellular ice. Cryobiology 1971;8:46–72.
18 Emmings FG. Cryotherapy for benign lesions of the oral cavity. J Oral Surg 1967;24:320–6.
19 Litvan GG. Mechanism of cryoinjury in biological systems. Cryobiology 1972;9:182–91.
20 Levitt J, Dear J. Role of membrane proteins in freezing: In: Wolstenholme GEW, O'Connor M, eds. The frozen cell. London Churchill JA 1970:149–74.
21 Gill W, Fraser J, Carter DC. Repeated freeze–thaw cycles in cryosurgery. Nature 1968;219:410–13.
22 Trump BF, Goldblatt J, Griffin CC, Waraudekar VS. Effect of freezing and thawing on the ultrastructure of mouse hepatic parenchymal cells. Lab Invest 1964;13:967–1002.
23 Rothenborg HW. ^{125}I-serum albumen assay of edema in rat skin with and without vasoconstriction after cryosurgery type freezing. Cryosurgery 1972;9:1–8.
24 Tarin D, Croft CB. Ultrastructural studies of wound healing in mouse skin, II. Dermo-epidermal interrelationships. J Anat 1970;106:79–91.
25 Zacarian SA. Histopathology of skin cancer following cryosurgery. Preliminary report. Int Surg 1970;54:255–63.